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Mycobacterial Antigens: a Review of Their Isolation, Chemistry, and Immunological Properties

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INTRODUCTION

Tuberculin antigens, derived from mycobacteria, have probably been more widely used by clinicians and immunologists than any other microbial antigen preparations. Moreover, there has probably been less understanding accompanying their use than has been true for any other widely used antigen preparation.

Extracts of mycobacteria—and tuberculins are nothing more than the crudest of such extracts—contain many antigens of varying chemical composition. Some of these antigens are probably species specific; others are known to be shared among many species and almost certainly contribute to the antigenic cross-reactivity commonly observed with tuberculin products. Yet immunochemists, epidemiologists, laboratory scientists, and clinicians who make frequent use of these materials often think of them as single antigens, as purified products, as standardized, or as specific, which they are not. Those who think critically feel a great need for isolated, purified, and standardized mycobacterial antigens, and many have turned to the fractionation of mycobacterial culture filtrates or cell extracts in hopes of isolating individual, purified mycobacterial antigens.

The last review devoted to the subject of mycobacterial antigens was published more than two decades ago by Boyden and Sorkin (33). In their introduction they commented that they were "struck by the lack of precise knowledge on the identity and biological significance of the antigens of the tubercle bacillus." They noted, for example, that the antigens of streptococci were much better known. The same statement could be made today, despite the application by many investigators of modern physicochemical methods to mycobacterial antigen purification and despite the exploding interest in the use of tuberculin antigens for the laboratory investigation of cell-mediated immunological phenomena as well as for the epidemiological investigation of mycobacterial diseases. Mycobacterial antigens were included among the many topics covered in the comprehensive recent review of the genus *Mycobacterium* by Barksdale and Kim (23). Their discussion is especially valuable in relation to serological taxonomy of mycobacteria. Immunological responses to mycobacteria were also considered.

In this review we will attempt to summarize the current state of knowledge concerning mycobacterial antigens. Emphasis will be placed on recent studies of water-soluble protein and polysaccharide antigens derived from culture filtrates and cell extracts. Mycobacterial lipids and their adjuvant properties will not be considered; they have been reviewed recently and comprehensively by Goren (88, 89). In part, the organization of the material presented in this review

takes cognizance of the great effort that has been expended on methods of fractionation. However, it is not the purpose of this presentation to review in detail specific techniques applicable to fractionation. Rather, the products of these methodologies will be considered, and the major immunochemical and immunobiological properties of isolated fractions will be discussed.

IDENTIFICATION AND NOMENCLATURE OF MYCOBACTERIAL ANTIGENS

It is probable that the number of individual mycobacterial antigens that can be identified and named is limited only by the effort which an investigator wishes to expend in the development of appropriate technology. However, any generally useful system of nomenclature and identification must also be readily and widely applicable; it should utilize small quantities of readily available reagents. At present only immunoelectrophoresis and acrylamide gel electrophoresis meet these criteria.

Many investigators have used precipitin techniques in gels for the analysis of mycobacterial antigens. Techniques used have included single- and two-dimensional immunoelectrophoresis. Nomenclatures based upon these methods have been used by individual investigators to classify mycobacteria and to identify individual antigens in mycobacterial products. However, none of these many distinct classification and nomenclature schemes has been used outside of the laboratory in which it was developed. Because of their limited availability, these many systems will not be considered further.

It was not until the work of Janicki and his collaborators from several independent laboratories in 1971 (50, 59, 100, 104) that a widely and readily applied system of identification and nomenclature for individual mycobacterial antigens became available. They prepared a reference unheated culture filtrate antigen from the virulent $H_{37}Rv$ strain of *Mycobacterium tuberculosis* and a homologous polyvalent goat antiserum suitable for use in immunoelectrophoresis. Subsequently additional materials were prepared (59) to provide a reference system for cell extracts as well as culture filtrates. Precipitin arcs obtained with these reagents were numbered as shown schematically in Fig. 1. The importance of standardized technique in the use of this reference system has been emphasized by Janicki and co-workers (100). Daniel et al. (59) have pointed out that the establishment of patterns of identity by the method of Osserman (158) or by other pattern modifications of standard immunoelectrophoresis (51) is necessary to relate precipitin arcs observed with nonreference materials to the reference nomenclature. They also demonstrated that different antigenic preparations and antisera gave somewhat different patterns, even when care was taken to duplicate the conditions of culture, immunization, and reagent preparation. These variations did not limit the usefulness of the reference materials, however, and additional supplies of standard reagents, including cell sonicate antigen from the same strain and homologous antiserum, were prepared and made available for distribution (59).

The immunoelectrophoresis reference reagents and nomenclature, sometimes designated as United States-Japan reagents because they were produced under sponsorship of the United States-Japan Cooperative Medical Sciences Program through the National Institute of Allergy and Infectious Diseases, have been used by a number of investigators to identify their products or to relate their methodologies to a common reference point (39, 48, 49, 51–54, 60, 107, 167, 204). This nomenclature is used throughout this review, and unless otherwise stated, Arabic numeral designation of antigens always refers to the nomenclature of Janicki and collaborators (104). Although the relatively simple immunoelectrophoresis technique of Janicki and his coworkers (59, 100, 104) is limited to the recognition of 11 major antigens, it offers the advantage of ready applicability with easily obtained materials.

Chaparas and Hedrick (43) used the reference antiserum to demonstrate the antigenic similarity of *M. bovis*, BCG strain, to *M. tuberculosis* and to show that no significant antigenic variation can be detected between various substrains of BCG from different laboratories. Subsequently Chaparas studied culture filtrates from 12 species of mycobacteria, using immunoelectrophoresis with reference antiserum (39). He concluded that no antigens detected by the reference antiserum are unique for *M. tuberculosis*, although several identifiable antigens had rather limited distribution among other mycobacteria. It should be noted, however, that Chaparas' identifications were based largely on position of antigen arcs and that his technique for establishing reactions of identity is one which may have given misleading results. In similar but more detailed studies using reference immunoelectrophoresis, Daniel and Todd found a much more limited distribution for several of the major antigens, notably antigen 5, which was limited to culture filtrates of *M. tuberculosis* and *M. bovis* among 14 species studied. Antigens 6, 7, and 8 were present in filtrates from the majority of species studied, and evidence was acquired that

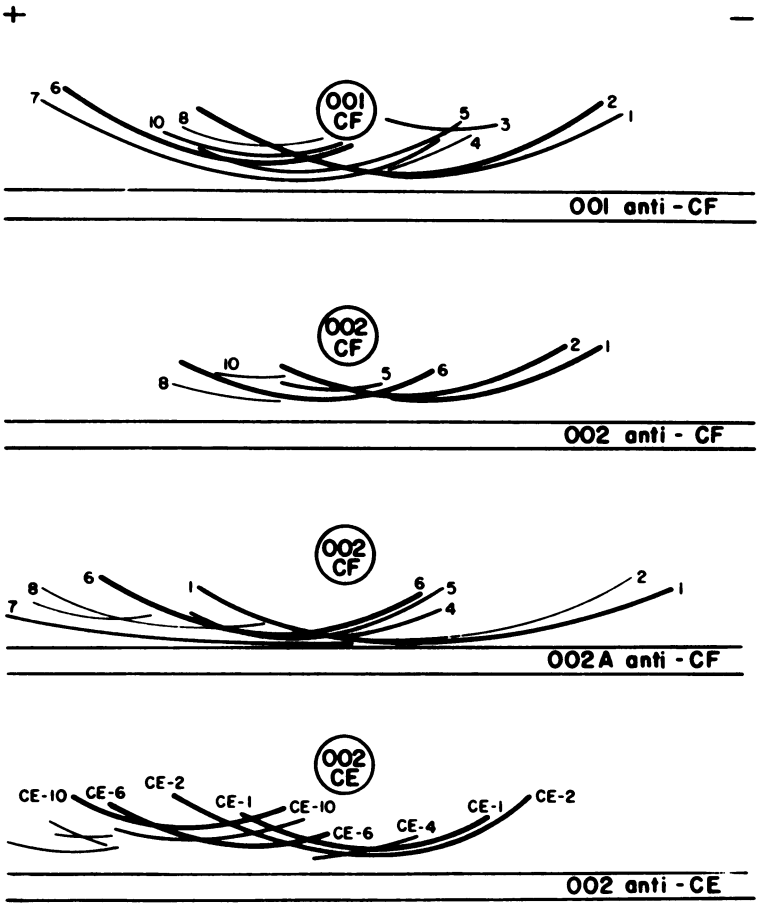

FIG. 1. *Schematic representation of the immunoelectrophoretic patterns obtained with reference antigens and antisera (59, 104) as presented by Daniel (50). The patterns obtained using culture filtrate (CF) and cell extract (CE) reference antigens, lots 001 and 002, with the homologous antisera are shown. Precipitin arcs are numbered as suggested by Janicki et al. (104). Reproduced with permission of the American Review of Respiratory Disease.*

both antigens 6 and 7 were present on molecules that contained both the widely distributed antigen and an additional antigenic determinant of more limited distribution (T. M. Daniel and L. S. Todd, submitted for publication). As noted below, there seems to be little question that antigens 1 and 2 are broadly distributed among the mycobacteria and probably other bacterial genera as well.

Polyacrylamide gel electrophoresis is a useful analytical tool for discriminating mycobacterial antigens. Provided that the physical conditions of acrylamide gel concentration, pH and buffer, and current flow are standardized, it provides a technique of powerful resolution that is readily adaptable to the identification and nomenclature of individual antigens. Moreover, the resolving power of gel electrophoretic techniques has been greatly increased by the two-dimensional procedures introduced by, among others, Augier and Augier-Gibory (9) and Wright and co-workers (202, 203). As illustrated in Fig. 2, gels may be stained for protein, and individual components may be identified by their characteristic mobilities and assigned R_f values. Antigenic components also can be identified in gels by reaction with appropriate antisera (57). Some of the bands recognizable by acrylamide gel electrophoresis have been related to the United States-Japan immunoelectrophoresis nomenclature. In a 7.0% gel at pH 9.5, antigen 5 purified by immunoabsorbent affinity chromatography (54) has an R_f of 0.61; physicochemically (58) or immunoabsorbent (T. M. Daniel and P. A. Anderson, unpublished data)-purified antigen 6 has an R_f of 0.68. Under these conditions, most poly-

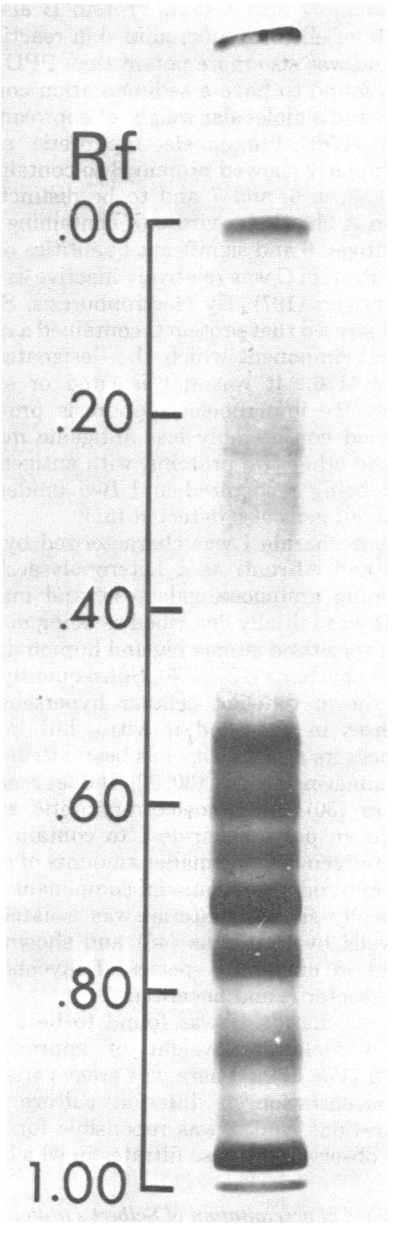

FIG. 2. *Electrophoresis of M. tuberculosis culture filtrate in 7.0% acrylamide gel at pH 9.5. The gel has been stained with Coomassie brilliant blue. Scale of R_f values is shown. A value of 1.00 is assigned to the bromophenol blue tracking dye.*

saccharide constituents of mycobacteria do not enter the acrylamide gel and remain stationary in the stacking gel. However, concanavalin A-purified arabinogalactan, which identifies with antigen 2 and is known to have a significant protein or peptide moiety (48), enters the gel and has an R_f value of 0.21. Although purified arabinogalactan is not detected with protein stains, whole-culture filtrate contains a band with a similar R_f which does stain with Coomassie brilliant blue (Fig. 2).

EARLY INVESTIGATIONS

In 1890, Robert Koch first reported the preparation of a product which he named "old tuberculin" (OT). It consisted of the concentrated sterile filtrate of autolyzed, heat-killed liquid cultures of *M. tuberculosis*. Later workers substituted synthetic media for that used by Koch and improved upon the methods of processing and concentrating OT. Tuberculin skin testing using such material was rapidly accepted into clinical and epidemiological practice in both veterinary and human medicine. However, nonspecific reactions to OT were soon encountered. Later it was recognized that the nonspecificity probably resulted from host environmental contact with other, often nonpathogenic species of mycobacteria that shared cross-reactive antigens with virulent mammalian tubercle bacilli. These early events have been elegantly reviewed by Edwards and Edwards (72). Their review from an epidemiological viewpoint nicely complements the microbiologically oriented reviews of Boyden (32), Boyden and Sorkin (33), and Barksdale and Kim (23).

In 1932, Florence Seibert first attempted to recover purified tuberculoprotein from OT by using trichloroacetic acid precipitation (175, 178), and in 1941 she described the preparation of a material termed "tuberculin purified protein derivative" (PPD) prepared from OT by repeated precipitation with ammonium sulfate at 50% saturation and neutral pH (177). In the manner of Robert Koch, the OT was derived from 8-week-old surface cultures of *M. tuberculosis*. A totally synthetic culture medium was used, and the cultures were steamed before filtration, a procedure that undoubtedly led to considerable heat denaturation of protein (174). Nevertheless, PPD rapidly became and remains the standard preparation for clinical tuberculin skin testing (6, 7, 72). OT also remains in use for this purpose, especially in the form of disposable multiple-puncture test units and veterinary products. Affronti prepared PPD materials from other species of mycobacteria (2). Such materials have been used in epidemological and clinical studies of tuberculin cross-reactivity (70–72). Further consideration of the extensive literature on skin testing with these PPD preparations or other similar products from various mycobacteria is beyond the scope of this review, except to point out that the clinical problems presented by cross-reactive antigens in the diagnosis of

various mycobacterial infections have not yet been resolved (65, 93).

PPD also has come into widespread use in the laboratory investigation of cell-mediated immunological responses. It has been widely used as a standard antigen for the study of the inhibition of macrophage migration (66, 80) and of blastogenesis in cultured lymphocytes (160, 173). However, the problems of specificity and potency standardization usually have not been considered in relation to the use of PPD preparations in these in vitro systems.

One of the first major attempts to purify isolated antigens from mycobacteria by chemical fractionation also was made by Florence Seibert, using precipitation with alcohol and acetic acid (176). For her studies, Seibert used 8-week-old surface cultures of *M. tuberculosis* grown on synthetic medium. Filtrates were prepared from these cultures without heating, thus avoiding the heat denaturation incurred in the classic preparation of OT and PPD. Most workers since have followed her lead and used unheated starting materials in their attempts to purify mycobacterial antigens. Seibert described four proteins and two polysaccharides, each distinct in physicochemical properties, which she designated proteins A, B, C, and D and polysaccharides I and II. Electrophoretic and solubility properties and conditions for isolation of Seibert's fractions as reported by Seibert (176) are summarized in Table 1. The relationship of these fractions to the immunoelectrophoretic reference system (104) is shown schematically in Fig. 3.

Protein A was capable of eliciting tuberculin skin reactions in man and was more potent than equal weights of PPD (197). It was recognized by Seibert that protein A contained at least two components, one of which was thought to be a protein with a molecular weight of 35,000 to 42,000 (176). However, immunoelectrophoretic studies by Daniel and Affronti demonstrated that protein A contained antigens 1, 2, 5, and 6 and probably also 4 (51). Protein B also was capable of eliciting tuberculin skin reactions in man and was also more potent than PPD (197). It was found to have a sedimentation constant of 2.0S and a molecular weight of approximately 20,000 (176). Immunoelectrophoretic studies (51) similarly showed protein B to contain antigens 1, 2, 5, 6, and 7 and to be distinct from protein A chiefly by virtue of containing much less antigen 6 and significant quantities of antigen 7. Protein C was relatively inactive as a skin test antigen (197). By electrophoresis, Seibert demonstrated that protein C contained a rapidly moving component which she designated protein D (176). It was not isolated or studied further. By immunoelectrophoresis protein C contained considerably less antigenic material than the other two proteins, with antigens 2, 6, and 7 being recognized and two unidentified anodal antigens also detected (51).

Polysaccharide I was characterized by Birnbaum and Affronti as a heteropolysaccharide containing arabinose, galactose, and mannose (28). It was initially described as being nonreactive in sensitized guinea pig and human delayed skin test systems (127, 176). Subsequently it has been shown to elicit cellular hypersensitivity responses in vivo and in vitro, but in these instances its antigenicity has been attributed to contaminating protein (30, 92). It does react with antisera (30). Immunoelectrophoretic analysis has shown polysaccharide I to contain principally antigen 2, with smaller amounts of antigen 1 and an unidentified anodal component (51). A chemically similar material was isolated from cell walls by Cummins (46) and shown to be present in numerous species of mycobacteria, corynebacteria, and nocardia.

Polysaccharide II was found to be a glucan with a molecular weight of approximately 100,000 (176, 180). There was great variation in its concentration in different culture filtrate preparations, and it was reponsible for opalescence observed in those filtrates in which it was

TABLE 1. *Electrophoretic mobilities, solubilities, and conditions of precipitation of Seibert's protein and polysaccharide fractions as reported by Seibert (176)*

Component	Mobility $\times 10^{-5}$ cm^2 $(V^{-1} s^{-1})^a$	Solubility at pH 3.8–4.7	Conditions for precipitation
Protein			
A	−3.4 to −3.8	Soluble	70% alcohol, pH 4.6
B	−5.4 to −6.4	Soluble	30% alcohol, pH 4.6
C	−6.1 to −7.3	Insoluble	Acetic acid, pH 4.0
D	−8.6	Insoluble	Acetic acid, pH 4.0
Polysaccharide			
I	−1.4 to −2.0	Soluble	95 to 100% alcohol
II	−1.4 to −1.6	Soluble	30% alcohol, pH 7.0

[a] Mobilities in phosphate buffer, pH 7.7, $\mu = 0.1$, and potential gradient of 9 to 10 V/cm.

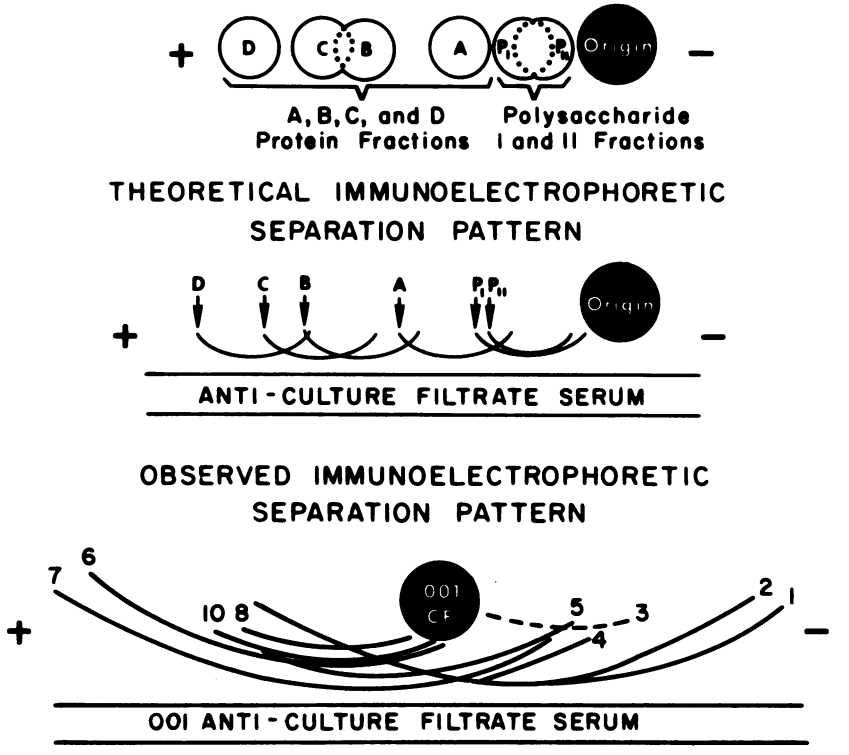

FIG. 3. *Schematic theoretical and observed electrophoretic and immunoelectrophoretic separation patterns for Seibert's protein and polysaccharide fractions. In the lower panel the origin has been shifted to compensate for electroendosmosis. The observed pattern shown is that obtained with lot 001 reagents prepared by Janicki and co-workers (104).*

present. It did not elicit delayed skin test reactions, but it did react with antisera (180). By immunoelectrophoresis it has been shown to be antigen 3 (51).

Other early workers also studied polysaccharides isolated from mycobacteria. In 1948, Haworth et al. isolated an antigenic dextrorotatory polysaccharide from defatted cell walls of *M. tuberculosis* by alkaline extraction and ethanol precipitation (91). They used their product to carry out the first structural studies undertaken of any mycobacterial antigenic constituent, and they concluded that their preparation had a highly branched structure composed of mannopyranose, arabinofuranose, and amino sugar units, with rhamnopyranose units forming terminal residues. A molecular weight of 12,000 was estimated. This product was later shown by Birnbaum and Affronti (28) to have serological identity with polysaccharide I of Seibert.

Recent studies, considered elsewhere in this review (see below), suggest that these early workers were dealing with D-arabino-D-galactan and D-arabino-D-mannan, with polysaccharide I containing both of these polysaccharides, and with a large-molecular-weight glucan of substantial purity.

SOURCE OF MYCOBACTERIAL ANTIGENS

There is no agreement upon the starting material best used for the isolation and purification of mycobacterial antigens. In many cases the choice of starting material has been dictated by the interests and needs of the individual investigator. From the viewpoint of the immunochemist, primary interest has resided in individual antigenic determinants of mycobacterial products, and their source has been less important. Recognizing that the carrier of these determinants may have important adjuvant or other modulating effects on the immune responses to these antigens, the source of these antigens has been of more importance to immunobiologists. Clearly, whatever source of antigens is used, it should be derived from cultures grown on a totally synthetic medium.

Sterile culture filtrates, usually of old, autolyzed cultures, have been used by a large number of workers. This source has the advantage of

ready availability in large quantity. Moreover, it is easily handled in aqueous systems and is relatively free of lipid and nucleic acid. The historical precedent for this source goes back to Koch, who first prepared OT from culture filtrate. Furthermore, findings obtained with materials of culture filtrate origin are more readily related to known properties of OT and PPD, which have been widely used in the laboratory and epidemiological investigation of tuberculin hypersensitivity, than are results obtained using materials extracted from whole cells.

In contrast, many investigators have preferred to work with extracts of whole mycobacterial cells or preparations of mycobacterial cell walls, often preliminarily extracted to decrease their lipid content. Certainly studies aimed at the identification of, for example, cell wall polysaccharides are best performed using such materials, although cell wall polysaccharides can be readily recovered from culture filtrate (48, 61). Whole cells have been disrupted by a variety of physical techniques, and whole organisms have been extracted in many ways. As reviewed by Janicki and his co-workers (107), mycobacteria have been disrupted by a variety of procedures, including mechanical grinding, pressure, and sonication. In fact, they showed that few antigenic differences could be detected in mycobacterial cell extracts prepared under different conditions. The study of Daniel et al. (59) demonstrated no gross qualitative differences between the antigenic composition of cell extracts and culture filtrates prepared from the same organisms under constant culture conditions. However, antigen 6 was found in large quantity in culture filtrate, whereas cell extracts were much richer in antigen 10.

It was recognized at an early date that the antigenic composition of filtrates of cultures grown under differing conditions varied significantly, with the variations being chiefly quantitative rather than qualitative (176, 180). Castelnuovo and co-workers felt that the antigenic composition of mycobacterial filtrates was determined principally by the age of the culture at the time of filtration (37), a view which was supported by the later work of Turcotte and Des Ormeaux (196) and that of Nagai and his colleagues (148). Kim et al. (111, 129) related antigen production to the medium used for culture as well as to the age and pH of the culture at the time of harvest. Age-related variation in cell extracts also has been described by others (64, 107, 187, 196). Incubation temperature has been reported to affect quantitatively but not qualitatively the antigen content of bacillary extracts and culture filtrates (124). Finally, variation in antigenic composition of culture filtrates grown under closely parallel cultural conditions for identical times also has been observed (107). This poorly understood variability emphasizes the importance of using common reference systems for the identification of antigens being studied in different laboratories and of using single large batches of antigen source materials in individual laboratories.

RECENT PHYSICOCHEMICAL STUDIES

Antigenic Proteins

During the past two decades, physicochemical fractionation methods that carry relatively little risk of denaturation have become available and have been used in attempts to isolate mycobacterial antigens. The methods used have included ion-exchange chromatography, molecular-exclusion chromatography, density gradient ultracentrifugation, isoelectric focusing, and zonal electrophoresis with or without the molecular sieving effect of an acrylamide gel supporting system. Salt or solvent solubility has often been used in combination with these techniques. A selected listing of preparative techniques is presented in Table 2. Despite the use of these techniques singly or in combinations by many investigators, our knowledge concerning the major protein antigens of mycobacteria remains largely descriptive and must always be viewed with reference to the particular isolation procedures used by individual workers.

Review of the many studies published concerning mycobacterial proteins reveals a complexity of antigens, few of which have been carefully characterized. It is probable that all cytoplasmic proteins of mycobacteria are antigenic in man and laboratory animals when proper conditions of immunization are used. Many such antigens probably possess species specificity. Since so many of the standard techniques of physical chemistry have been applied to the purification of mycobacterial proteins, it is convenient to consider the literature on this subject by grouping studies according to the method of fractionation used. In many cases, of course, different investigators may have been working with the same protein, and in most cases the available data do not allow comparisons between investigations carried out in different laboratories. It should also be realized that most attempts to purify mycobacterial proteins have not excluded polysaccharides that may be derived either from cell wall or cell cytoplasm, and many so-called protein fractions have high polysaccharide content.

Ion-exchange chromatography with diethyl-

TABLE 2. *Selected major physicochemical purifications of mycobacterial antigens*

Investigator	Methods	Products
Seibert (176)	Ethanol-acetic acid precipitation	Proteins A, B, C, and D and polysaccharides I and II; none antigenically pure (51)
Kniker and LaBorde (114)	DEAE-cellulose ion-exchange chromatography	Protein- and polysaccharide-rich antigenic fractions; none antigenically pure
Baer and Chaparas (19, 20, 40)	Molecular-exclusion chromatography	Protein- and polysaccharide-rich antigenic fractions; none antigenically pure
Janicki et al. (102)	Paper curtain electrophoresis	Protein- and polysaccharide-rich antigenic fractions; none antigenically pure
Roszman et al. (172); Minden and Farr (133)	Acrylamide gel electrophoresis	Small quantities of isolated, purified antigenic proteins
Yoneda and Fukui (212); Daniel and Ferguson (58); Kuwabara (117, 118)	Serial ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, zonal electrophoresis	Highly purified antigenic proteins with low yields
Azuma et al. (12); Yamamura et al. (206); Misaki et al. (138–140, 142)	Alkaline extraction, ethanol precipitation, ion-exchange chromatography	Highly purified antigenic arabinogalactan and arabinomannan; nonantigenic mannan and glucan
Daniel (48)	Concanavalin A affinity chromatography	Purified antigenic arabinogalactan and arabinomannan (61)
Daniel and Anderson (52–54)	Immunoabsorbent affinity chromatography	Highly purified protein antigen 5 (53, 54) and antigen 6

aminoethyl (DEAE)-cellulose was used as a sole fractionation procedure by Kniker and La Borde (114). Stepwise increases in ionic strength with decreasing pH led to the elution of several antigen-containing peaks when culture filtrates of *M. tuberculosis* and of three other mycobacteria were chromatographed. As illustrated in Fig. 4, the chromatograms showed excellent resolution. Three major peaks (designated wash, A, and BC) were obtained; several minor peaks were recovered, and antigens also were identified in the interpeak fractions. Serological analysis revealed that most fractions contained more than one antigen. Using a battery of rabbit antisera, the species specificity of these antigens was studied. Nonspecific antigens were broadly distributed throughout the eluted fractions, although specific and group antigens tended to concentrate in the fractions eluted at high salt concentrations. Subsequently Kniker used the same methods to study Seibert proteins A, B, and C (113). Each was found to have a predominant elution peak; however, each also was found to have multiple antigens. The first eluted peak, designated the wash fraction because it contained material that did not absorb to the resin, was not identified with any of the three Seibert proteins and probably contained principally polysaccharides. Protein A of Seibert was associated with the second major peak eluted when whole filtrate was chromatographed. Proteins B and C were both associated with the third major peak, requiring high salt concentrations for elution, and protein B preceded protein C within this broad peak. When whole-culture filtrate was chromatographed, the optical absorbancy at 260 nm exceeded that at 280 nm throughout the chromatogram. This was especially striking in the first peak, thought to contain principally polysaccharides. Using the Seibert proteins, proteins A and C were eluted in major peaks with absorbancy at 280 nm greater than that at 260 nm. This finding suggests that a significant decrease in polysaccharide contamination was achieved by the preliminary use of the Seibert ethanol and acetic acid fractionation procedure.

Numerous other reports document the utility of ion-exchange chromatography in fractionating mycobacterial products, although no more than partial purification has been achieved by this single technique. Bennedsen subjected an unheated culture filtrate and a saline cell extract of *M. tuberculosis* to chromatography on DEAE-Sephadex after preliminary precipitation with 80% saturated ammonium sulfate (26). As expected, his chromatograms differed from those of Kniker (113, 114) in that the first eluted peak was much smaller. Some evidence for species specificity was demonstrated for the fraction that he designated as number 2, which was eluted in the first interpeak zone; rabbit antiserum raised with this fraction reacted by fluorescent-antibody techniques only with *M. tuberculosis* and *M. bovis*. This serum when used in

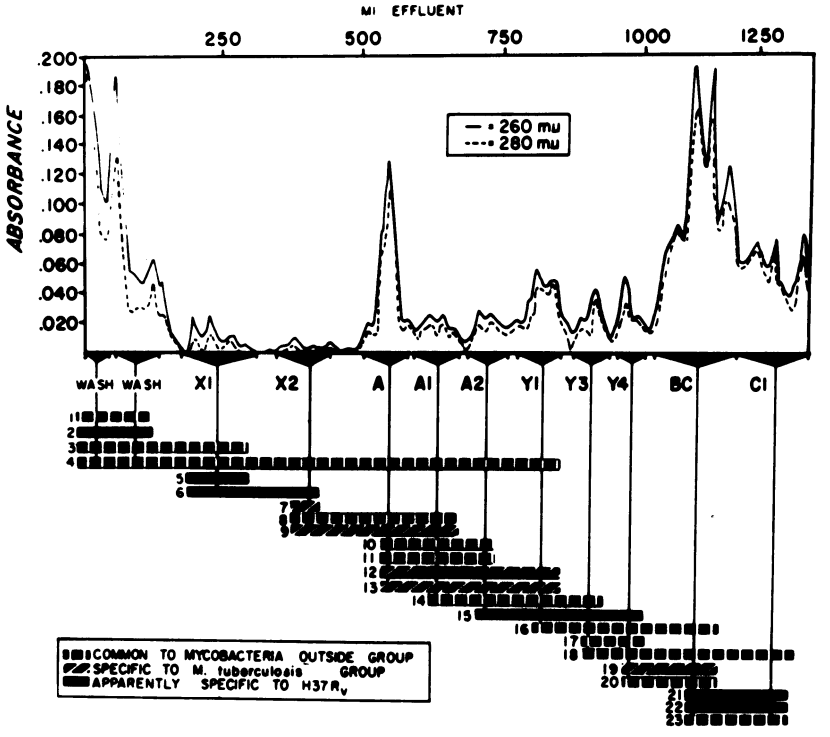

FIG. 4. *Chromatographic fractionation of M. tuberculosis culture filtrate on DEAE-cellulose as achieved by Knicker and LaBorde (114). Antigen content detected by precipitin analysis in gel is shown beneath the chromatogram. Reproducted with permission of the American Review of Respiratory Disease.*

immunoelectrophoresis against whole saline extract gave a single cathodal precipitin band, suggesting that it was reacting with a polysaccharide. Because they were so readily extracted with saline, Bennedsen considered his fractions to contain surface antigens.

Glenchur and his co-workers used DEAE-cellulose chromatography in a series of studies of pressure cell extracts and culture filtrates of *M. tuberculosis* (82–85). They initially subjected their materials to Sephadex G-25 chromatography and showed that antigenic activity was located in the first eluted, exclusion peak. This peak almost certainly contained all of the proteins and polysaccharides present in their starting materials. Their studies demonstrated that antigens reactive with antisera and effective in tuberculin skin tests were eluted in all of the DEAE-cellulose fractions. No fractions contained isolated antigens. One fraction, eluted at lowest pH and highest salt concentration, elicited an early skin reaction in tuberculous patients which was not seen in control subjects (83).

Diena and co-workers used DEAE-cellulose chromatography to fractionate a cell sonicate prepared from 2-week-old cultures of *M. bovis*, BCG strain (68). Their chromatograms showed an excellent degree of resolution. As expected, peaks eluted early were largely polysaccharide and late peaks were principally protein. Further immunochemical or serological analysis of the eluted peaks was not done. However, the carbohydrate eluted in the first peak did elicit immediate but not delayed skin test reactions in sensitized guinea pigs. The last recovered peak, which contained 88% protein, was the most potent skin test antigen. Heterologously sensitized animals were also tested with each fraction. A statistical analysis of the results was not presented, but the tabulated data implied that some degree of specificity resided in those fractions with the highest protein content.

Navalkar and his colleagues prepared a cell extract from *M. leprae* isolated from lepromas (150) and fractionated this material by DEAE-cellulose chromatography (151). Three fractions, designated A, B, and C, were obtained. The elution chromatogram was not presented in their publication, prohibiting comparison with the studies of Kniker and others. Fractions A and B were found to contain multiple protein and polysaccharide antigens; only a single protein antigen could be identified in fraction C. When

used in delayed skin test and passive cutaneous anaphylaxis studies in guinea pigs sensitized with *M. leprae* and other mycobacteria, species specificity was found in fraction C but not in fractions A and B.

DEAE-Sephadex chromatography was used by Ortiz-Ortiz et al. to fractionate acid-precipitable protein from pressure cell bacillary extracts of *M. bovis*, BCG strain (156). Gradient elution was used, and moderate resolution was achieved. Each major fraction was capable of eliciting delayed skin test reactions in sensitized guinea pigs. Pickett and collaborators obtained good resolution with DEAE-cellulose chromatography of ammonium sulfate fractions of *M. tuberculosis* culture filtrates and pressure cell extracts (162). Analysis by immunoelectrophoresis demonstrated multiple antigens in most of the final fractions. Castelnuovo and co-workers attempted DEAE-cellulose chromatography of *M. phlei* pressure cell bacillary extract and were unable to obtain satisfactory resolution (38). Poor resolution also was reported by Rhodes (164). Carboxymethyl cellulose ion-exchange chromatography was used by Lind to fractionate *M. tuberculosis* culture filtrate (122). When studied serologically, his fractions contained multiple antigens which overlapped considerably from one fraction to another.

None of the ion-exchange chromatographic studies cited above led to the recovery of fractions containing single or specific antigens, although some degree of purification was achieved in every case. In contrast, separation of polysaccharides from proteins can be readily obtained because polysaccharides are uncharged and are not absorbed by the resin.

Molecular-exclusion chromatography with high-porosity Sephadex G-100 and G-200 was compared with DEAE-cellulose chromatography by Castelnuovo (38). Her published chromatograms do not show good resolution, but she felt that better results were obtained with Sephadex than with DEAE-cellulose. Moderate resolution was reported by Lind (123) when Sephadex G-100 was used to fractionate a concentrated filtrate of *M. avium*, but no single-antigen fractions were recovered. Hornez et al. used Sephadex G-150 to isolate active L-asparaginase from *M. phlei* (95).

Low-porosity Sephadex G-25 and G-50 were used by Baer and Chaparas to fractionate the acid-soluble and -insoluble, nondialyzable components of culture filtrates of *M. bovis*, BCG strain (18–20, 40, 42, 86). The acid-insoluble material was resolved by this procedure into two pools, both high in protein content, both containing multiple antigenic constituents when studied with appropriate antisera, and both capable of eliciting delayed skin test reactions in homologously and heterologously sensitized guinea pigs. The acid-soluble material was resolved into three fractions. The first eluted was largely polysaccharide. It elicited delayed skin test reactions and inhibited migration of macrophages from sensitized animals. These properties could not be eliminated by treatment with Pronase and trypsin (20, 86). This material did not stimulate mitogenesis in cultured guinea pig lymphocytes (44, 45). When tested in man at one-fifth or one-sixtieth the dose used in guinea pigs, this polysaccharide did not elicit delayed skin test reactions in most tuberculin reactors (170). The remaining two fractions, which were approximately 50% polysaccharide, elicited tuberculin skin test reactions in sensitized guinea pigs, inhibited macrophage migration, and stimulated mitogenesis; their antigenicity was reduced by enzyme treatment (45). All three fractions were found to contain multiple antigens when assayed serologically.

Other preparative approaches based on molecular-size differences, such as density gradient ultracentrifugation, have had only limited application. Roos and co-workers used this method to fractionate a bacillary extract of *M. phlei*, but did not recover individual antigens (169).

There have been numerous attempts to apply electrophoresis for fractionation of mycobacterial antigens. As early as 1938, Seibert and her co-workers (179) were partially successful in fractionating tuberculin in the Tiselius apparatus. Seibert and Watson (181) subsequently isolated several polysaccharide fractions and nucleic acid from tuberculin by this method. More recently, preparative zonal methods have been used with a variety of supporting media. Rhodes et al. used zonal electrophoresis on glass beads to partially purify the polysaccharide hemosensitin of *M. tuberculosis* culture filtrate (165). Merkal used continuous-flow paper curtain zonal electrophoresis to fractionate further materials precipitated from culture filtrate of *M. paratuberculosis* with ethanol and acetic acid (130). More recently, Janicki and his collaborators used the same technique to fractionate culture filtrates of *M. tuberculosis* (102) and then subjected the fractions obtained to subsequent study and analysis. A polysaccharide-rich cathodal pool of fractions contained antigens 1, 2, and 3, while anodal pools were largely proteinaceous. They concluded that zonal electrophoresis as a single technique was unlikely to yield isolated antigenic constituents, with the possible exception of polysaccharides. Subsequent studies using these fractions (B. W. Janicki, unpublished data) have shown that individual antigens are heterogeneous with respect to molecular

charge or size. Re-electrophoresis of individual electrophoretic fractions clearly separates antigens that are immunologically identical but have reproducibly distinct electrophoretic mobilities.

Janicki's polysaccharide pool contained the principal antigen reacting in immunodiffusion systems with the sera of tuberculous patients (105) and responsible for tuberculin shock in sensitized animals (101). This pool did not stimulate mitogenesis in cultured lymphocytes from tuberculous patients (103). Daniel and Hinz made similar observations, using polysaccharides purified by ion-exchange chromatography (60). As noted above, a dichotomy between delayed skin test reactivity and macrophage migration inhibition on one hand and lymphocyte mitogenicity on the other has been observed in guinea pigs by Chaparas and co-workers (44, 45), using Sephadex-purified mycobacterial polysaccharide. It seems possible that some mycobacterial polysaccharides may have distinctive antigenic properties that cause them to react differently in different hosts, under differing conditions of immunization, or in different assay systems. This is probably not true for protein-containing fractions (60, 103), and a close correlation between delayed skin test reactions and lymphocyte mitogenesis has been demonstrated with PPD in healthy tuberculin-positive persons (106, 128) and in patients with tuberculosis (94, 110). However, these antigenic dichotomies are not necessarily restricted to mycobacterial polysaccharide antigens. Janicki et al. found a poor correlation between cultured lymphocyte thymidine incorporation and delayed skin test reactions in patients with active tuberculosis (106). Absence of lymphocyte stimulation in appropriately sensitized guinea pigs has been reported, using well-characterized haptenic peptides derived from tobacco mosaic virus protein (185) and from clostridial ferredoxin (198). Failure to stimulate lymphocyte mitogenesis might be explained as a specific defect in antigenicity; alternatively, it might represent an antigen-related suppressor effect. Such suppressor influences may specifically affect proliferation-dependent assays (112). Since the production of migration inhibition factor may be independent of lymphocyte proliferation (168), the dichotomy may reflect a differential effect on immunologically reactive subsets of lymphocytes.

Several investigators have combined two or more physicochemical separation techniques serially. This approach has yielded preparations in which only single antigenic constituents can be identified, although the yield of these materials has generally been very small. Yoneda et al. sequentially subjected unheated filtrates of *M. tuberculosis* to ammonium sulfate precipitation at 30, 50, or 80% saturation; zonal electrophoreses on starch; and ion-exchange chromatography using DEAE-cellulose or hydroxylapatite columns (77, 79, 210–213). From this procedure many fractions were obtained. Two, designated alpha and beta (mpt-1 and mpt-3, respectively, in early publications), were studied extensively. Both were thought to be proteins with little carbohydrate content and to be major components of crude culture filtrate. They could not be identified with Seibert protein A, B, or C. They were both completely precipitable with antiserum. Beta antigen was present in *M. tuberculosis, M. bovis,* and *M. microti*; it was not present in nine other species of mycobacteria examined. Alpha antigen also was present in *M. tuberculosis, M. bovis,* and *M. microti.* A cross-reacting material with partial identity with alpha protein was found in *M. avium, M. ulcerans, M. paratuberculosis, M. marinum* (identified as *M. balnei*), and *M. lepraemurium.* Alpha protein was not found in *M. fortuitum, M. smegmatis,* or *M. phlei* (78, 213).

Daniel and Ferguson combined ammonium sulfate precipitation, gel filtration with high-porosity P-300 acrylamide gel, DEAE-cellulose ion-exchange chromatography, and zonal electrophoresis on polyvinyl chloride (58). They isolated two proteins, designated a_1 and a_2, from culture filtrates of *M. tuberculosis,* both of which were estimated to have sedimentation constants of 2.8S and molecular weights of 45,000 to 48,000. They could be separated electrophoretically. In subsequent studies (60), a_2 was shown to identify with antigen 6. Both a_1 and a_2 were shown to elicit tuberculin skin tests in sensitized guinea pigs and to induce mitogenesis in cultured lymphocytes of some but not all tuberculin-positive human donors (60).

Castelnuovo and Duncan combined high-porosity gel filtration with ion-exchange chromatography to obtain sharp separation of *M. phlei* antigens identified by their antiserum (36). Kasik and his colleagues used the combination of gel filtration and DEAE-cellulose chromatography to isolate mycobacterial beta-lactamase (109). This product was enzymatically active and was antigenically distinct from beta-lactamases isolated from other bacterial genera when tested with appropriate antisera. Diaz and Wayne purified *M. tuberculosis* catalase by ion-exchange chromatography and gel filtration (67) and then prepared a specific rabbit antiserum to this enzyme, which was used to study the catalase of several mycobacterial species (200). The antibody precipitated but did not inactivate the enzyme from mycobacterial cell extracts. Catalases from all other mycobacterial species were incompletely bound by the antibody, suggesting

small variations in the amino acid sequence at the antigenic determinant sites. Catalases from several mycobacterial species also were studied by Stavri and Stavri, using an immunoelectrophoretic technique with subsequent demonstration of catalase activity in the immune precipitates (188). In this fashion species-related electrophoretic heterogeneity was demonstrated among mycobacterial catalases without actually purifying the enzyme. Similar techniques were used by Lipinska and Rcuzidlo to study mycobacterial peroxidase (125), although studies by these workers were confined to two BCG substrains.

Abe and his collaborators used zone electrophoresis and Sephadex G-200 gel filtration to fractionate a soluble extract of lepromatous nodules (1). Their data suggested that a polysaccharide antigen of *M. leprae* is shared with other mycobacteria and that a major protein, which also can be extracted from lepromata, is antigenic, is derived from *M. leprae* bacilli, and is not shared with other mycobacteria.

The combination of gel filtration with Sephadex G-200, DEAE-Sephadex ion-exchange chromatography, and isoelectric focusing was used to fractionate culture filtrate of *M. tuberculosis* by Moulton et al. (146). They obtained nine fractions, four of which had precipitinogens when studied by immunodiffusion with rabbit antisera, two giving only single lines. Only one fraction was obtained which elicited delayed skin test reactions in sensitized guinea pigs. With this fraction reactions were observed in control animals, although there was a statistically significant difference in mean reaction diameter between sensitized and unsensitized animals.

Electrophoresis on acrylamide gels is a fractionation method that combines zonal electrophoresis with molecular sieving provided by the supporting gel. The degree of cross-linking of the gel can be varied by changing the acrylamide concentration, resulting in differing sieving effects. By this method, extremely high resolution is possible. Affronti et al. first applied acrylamide gel electrophoresis to the fractionation of mycobacterial antigens (4). Unheated culture filtrate of *M. tuberculosis* and Seibert fractions were studied, and R_f values for major components were determined. As applied, the method was an analytic one, and fractions were not recovered for further study.

Roszman and his co-workers (172) used this approach to fractionate culture filtrates of several mycobacterial species. Upon completion of electrophoresis, gels were sliced using a stained gel as a guide, and the slices were extracted with buffer. Sufficient quantities of materials were recovered in this way to allow study with antisera, and in some eluates only single antigens were identified by immunodiffusion. One very anodal antigen was thought to be specific for *M. bovis* and *M. tuberculosis* on the basis of immunodiffusion reactions. A subsequent preliminary report from the same laboratory by Fauser and co-workers (75) indicated this antigen could also be isolated by hydroxylapatite ion-exchange chromatography, but further studies of this antigen have not been published.

Acrylamide gel electrophoresis was applied by Dietz and collaborators (69) to the fractionation of cell sonicates and culture filtrates of *M. kansasii*. From the former they recovered fractions that were used to skin test sensitized guinea pigs. Positive reactions were observed, but the purified fractions were less potent than a PPD prepared from *M. kansasii*. Castelnuovo and coworkers (38) used elution from acrylamide gels to recover small amounts of antigenic materials from a cell pressure extract of *M. phlei*. Similarly Bennedsen (26) recovered *M. tuberculosis* antigens from acrylamide gels; serological evidence of specificity was found for one very anodal component. Nassau and Nelstrop used acrylamide gel electrophoresis to recover four antigens of *M. tuberculosis* which they felt were probably species specific (149). However, their report gives relatively little data upon which their impression of specificity can be evaluated.

Modifications in the basic acrylamide gel electrophoretic technique have increased its resolving power. Augier and Augier-Gibory (9) and Wright and co-workers (202) have introduced two-dimensional procedures for analytic purposes. Affronti et al. (5) introduced the use of discontinuous gel gradients in this technique. Finally, Minden and Farr (133) developed a preparative scale method for using gradient acrylamide gel electrophoresis to fractionate mycobacterial antigens. Using this method, they isolated a rapidly migrating glycoprotein with a molecular weight of 9,000 to 12,000 that was soluble in 50% saturated ammonium sulfate (133). It was antigenic as determined by binding to antisera, and some evidence of species specificity was observed. Its antigenicity was destroyed by treatment with Pronase.

A preparative method similar to that of Minden and Farr (133) was applied in a collaborative study by Janicki, Wright, Daniel, Chaparas, Good, and Goldstein (unpublished data). Unheated *M. tuberculosis* culture filtrate, previously described as lot 002 (59), was fractionated by acrylamide gel electrophoresis with fractions recovered by elution from gel slices. Each fraction was studied serologically to identify its major components with the United States-Japan reference nomenclature, was studied in homol-

ogously and heterologously sensitized guinea pigs to determine its tuberculin skin test potency and specificity, and was studied in human lymphocyte cultures to determine its ability to stimulate thymidine incorporation. Both the major in vivo and in vitro reactivity and the major antigenic specificity were found to be associated with fractions containing relatively large amounts of antigens 5 and 6. The resolution obtained was not great enough to allow choice between these two antigens with respect to either antigenic reactivity or specificity. Fractions containing tuberculopolysaccharide antigens 1, 2, and 3 failed to induce significant blastogenic responses in human lymphocytes and evoked nonspecific skin reactions in sensitized guinea pigs. Also, no significant antigenicity was found in extremely anodal fractions, suggesting that the primary binding antigen of Minden and Farr (133) may not react in skin test and cultured lymphocyte test systems.

The same group of investigators (S. D. Chaparas et al., unpublished data) also applied preparative gradient acrylamide gel electrophoresis to a sonicate of *M. tuberculosis* cells. Findings were similar to those observed for culture filtrate, with maximum antigenic reactivity and species specificity found in fractions containing antigens 5 and 6. Laguerre and Turcotte (120) applied gradient acrylamide gel electrophoresis to the fractionation of pressure cell extract of *M. bovis,* BCG strain cells after a preliminary gel filtration fractionation. They also found maximum delayed skin test reactivity in the central portion of their gels, and review of their published electrophoretic patterns suggests that the observed reactivity may well have been associated with antigens 5 and 6.

PPD; Proteins from Heated Materials

The early work of Seibert (174) demonstrated that the heating of mycobacterial culture filtrates led to a significant loss by denaturation of antigenic protein. Most of the work considered in this review has dealt with antigens derived from unheated sources. In the same study, Seibert also demonstrated that some antigens were not subject to heat denaturation, and, indeed, PPD is made from heat-killed cultures. It is worthwhile, then, to review the somewhat limited information available concerning antigens from heated mycobacterial sources.

As mentioned previously, PPD represents simply the precipitate resulting when OT (from heat-killed cultures) is brought to 50% saturation with ammonium sulfate. In our hands (Daniel, unpublished data), the most readily identified antigenic constituents of PPD are cell wall polysaccharides, antigens 1 and 2; no other antigens are identified with reference antisera. Affronti and his co-workers (3) demonstrated that many discrete components could be recognized in PPD by the use of acrylamide gel electrophoresis. The gels were sliced and segments were eluted for recovery of antigenic fractions. Greatest skin reactivity was found associated with low-molecular-weight components.

Moulton and colleagues (147) subjected PPD to gel filtration with Sephadex G-200. An excluded peak with minimal delayed skin test antigenicity in sensitized guinea pigs appeared by immunoelectrophoresis to be similar to that usually seen with antigen 3, which is known to identify with the large-molecular-weight glucan termed polysaccharide II by Seibert. The partially retarded material, also capable of eliciting delayed skin tests in guinea pigs, gave an immunoelectrophoretic pattern that almost certainly identifies it as a mixture of arabinomannan (antigen 1) and arabinogalactan (antigen 2). In the small-molecular-weight fraction, a single anodal antigen was identified. Maximum species specificity rested with this fraction.

Studies by Glenchur and his co-workers (82) demonstrated that Sephadex G-25 gel filtration followed by DEAE-cellulose chromatography resolved PPD into multiple constituents. On the basis of parallel studies of fractions similarly prepared from unheated materials, they concluded that tuberculin activity resided chiefly with high-molecular-weight proteins. Skin test data for fractions of PPD were not given.

Nagai and his co-workers (148) fractionated the filtrate of autoclaved cultures of *M. tuberculosis,* using sequential ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and preparative acrylamide gel electrophoresis. Five isolated proteins were obtained, all with molecular weights of approximately 10,000 and all capable of eliciting delayed skin test reactions in homologously sensitized guinea pigs. Essentially negative reactions were observed in animals sensitized with *M. smegmatis.*

Kuwabara and Tsumita isolated a protein from *M. tuberculosis* that had a sedimentation constant of 1.73S and a molecular weight of 9,700 and for which they determined the complete amino acid sequence (117–119). Autoclaved, acetone-dried cells were ground, extracted with low-ionic-strength buffer, and treated with deoxyribonuclease and ribonuclease. The treated extract was chromatographed twice on DEAE-cellulose, and the major peak was then chromatographed on Sephadex G-200. Finally the protein was crystallized from solution with 85% saturated ammonium sulfate and 50% acetone. The recovered protein represented

about 1.5% of the protein content of the original cell extract. It was skin test reactive, and on a per unit of weight basis was approximately one-tenth as potent as PPD in sensitized guinea pigs. Only one of many peptides generated by enzymatic digestion had tuberculin activity, and large amounts of it were required to elicit delayed skin test reactions. It had an amino acid sequence of Asn-Gly-Ser-Gln-Met-Arg.

The work of Kuwabara and Tsumita represents the first structural analysis of any tuberculin-active protein. However, it is difficult to relate their findings to the work of most other investigators because the starting material was prepared from heat-killed bacilli. It should be noted, of course, that PPD is similarly prepared from heat-killed cultures. The extensive exposure to organic solvents incurred in the purification process also might be expected to cause denaturation of some protein constituents of mycobacteria. A sample of Kuwabara's material was analyzed with reference antiserum by one of us (Daniel, unpublished data). It was found to contain tuberculopolysaccharide antigens 1 and 2. Several other minor components that could not be identified were also detected. It is possible that the reference antiserum used for this analysis did not react with the major component of Kuwabara's preparation.

Taken collectively, the fractionation studies that have been carried out on PPD would suggest that this product is a mixture of antigenic constituents, most of them having molecular weights in the range of 10,000. The major purification step in the preparation of PPD is probably the coagulation of many heat-labile components during the heat killing of the cultures from which it is prepared. Substantial amounts of polysaccharide are probably present in most preparations of PPD, and it is likely that there is considerable variation in the composition of differing lots of PPD.

Antigenic Polysaccharides

It has already been noted that chemical methods were used by Seibert, Stacey, Kent, Cummins, Affronti, and other pioneering investigators to extract polysaccharides from mycobacteria. Their early contributions have been reviewed by Stacey (186). Other workers used physicochemical techniques to isolate polysaccharides (102). In some cases moderately high purity of product was obtained, and many of these products were found to be tuberculin active. In recent years many investigators have continued to study mycobacterial polysaccharides, and attention has been directed primarily at cell wall components. Mycobacterial cell walls are rich in peptidoglycan. Much attention has been devoted to the study of this material and to the lipid complexed to it. Crude preparations of peptidoglycan elicit delayed skin reactions in sensitized guinea pigs but with much less potency than that of PPD (171). Such antigenic preparations have been shown to have some degree of species specificity (171). The adjuvant properties of lipid containing fractions of mycobacterial cell walls are well known (88, 121) and will not be considered here. Polysaccharides and the peptides important for linkages between polysaccharide moieties have been purified from cell walls. These purifications have usually involved alkaline extraction of cell walls or cells that first had been delipidated with organic solvents, followed by precipitation with organic solvents. Further purification has sometimes been achieved with ion-exchange chromatography. Proteolytic enzymes have been used to remove remaining peptide or protein components. Those with specific interest in mycobacterial cell walls should refer to the recent review by Barksdale and Kim (23), the comprehensive review by Lederer (121), the biochemical and immunochemical investigations of Kotani and his colleagues (115, 116), and the ultrastructural studies of Imaeda and co-workers (97).

The work of Misaki, Azuma, Yamamura, and others has resulted in the recovery of protein-free polysaccharides of four principal types, arabinogalactans, arabinomannans, mannans, and glucans, from both mycobacterial culture filtrates and cell wall extracts (10–17, 138–144, 205–207). There is some evidence that mycobacteria may also contain arabinans (61). Some of these types may be represented by more than one structurally distinct polysaccharide. Figures 5 and 6 present the currently accepted structural formulas for three major polysaccharides. All of these polysaccharides are dextrorotatory. The arabinomannans and arabinogalactans have been shown to possess antigenic reactivity, and, as noted below, it may be limited to immediate hypersensitivity phenomena. Arabinogalactan is a major, structurally important cell wall constituent (141–143). Extensive carbohydrate analysis has been carried out on mycobacterial D-arabino-D-galactan with elucidation of its general structure (141, 142), relationship to the cell wall (108), and identification of its major antigenic determinant (142).

The structures of mycobacterial arabinomannan (139, 140, 154) and mannan (138, 139) have been recently elucidated. Arabinomannans appear to have a common central core (139). One major side chain is an arabinose-containing oligosaccharide that is similar to the antigenic site-containing side chain of arabinogalactan (142). Other side chains contain principally mannose

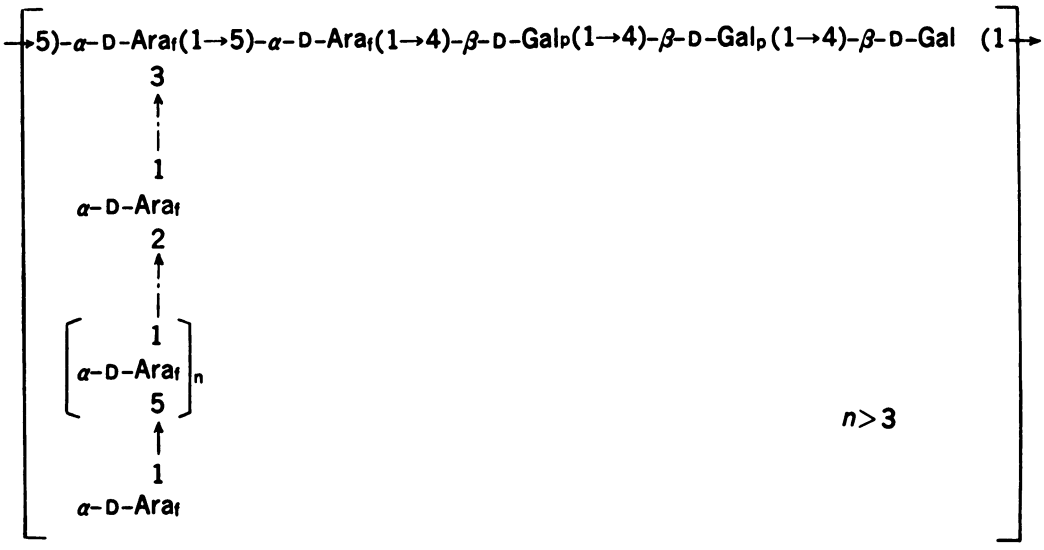

FIG. 5. *General structure of mycobacterial arabinogalactan as proposed by Misaki et al. (142). Arabinose (Ara) and galactose (Gal) are indicated as in their furanose (f) or pyrannose (p) forms, and the linkages are shown. If the* D-*galactose residues are in the furanose form, the linkage should be (1→5). Reproduced with permission of the Journal of Biochemistry.*

and probably vary from species to species (139). The central core of mycobacterial mannan is similar to a portion of the core of arabinomannan (139). Mycobacterial glucan and mannan are derived from middle or inner layers of cell wall (97) and generally do not elicit immune responses in animals sensitized with whole organisms. An exception may be Seibert's polysaccharide II, a macromolecular glucan (180), known to precipitate with specific antisera (51, 180) and to identify with reference antigen 3 (104).

There is a high degree of chemical similarity or identity among polysaccharides isolated from various mycobacteria. *M. tuberculosis, M. bovis, M. kansasii, M. smegmatis,* and *M. phlei* have all been shown to yield similar polysaccharides (10, 28, 142). In fact, corynebacteria and nocardia have been shown to contain polysaccharides similar to those found in mycobacteria (10, 28, 46, 142). Antigenic identity correlating well with the chemical similarity has also been demonstrated for arabinogalactan derived from these organisms (10, 28–30, 46, 142). Arabinomannan may contain specific antigenic determinants (139).

As mentioned earlier, antigenicity in polysaccharide preparations derived from mycobacteria has often been attributed to contaminating proteins or peptide moieties. However, as noted below, there is convincing evidence that some antigenic sites, especially those reactive with antisera and capable of eliciting immediate skin reactions, are carbohydrate in nature and independent of peptide or protein present in the polysaccharide preparations. In this regard, it should be recognized that the exposure to alkaline extraction conditions and organic solvents incurred in the preparation of many recently studied polysaccharide fractions would be expected to produce denaturation of most contaminating proteins.

Early skin test reactions were demonstrated by Misaki et al. when arabinogalactan was used to test sensitized guinea pigs (142). The polysaccharide also reacted with antisera in precipitin, passive hemagglutination, and complement fixation tests. Delayed skin test reactions could not be elicited in sensitized guinea pigs with this material. The work of these authors and of Kotani and his collaborators (115) demonstrated that, when treated with an enzyme of streptomyces origin capable of degrading arabinogalactans, this product lost its antigenicity and yielded a series of D-arabino-oligosaccharides and a degraded arabinogalactan backbone with a molar ratio of 2:5.4. One of these oligosaccharides, a D-arabinohexaose containing both α-(1→5)- and α-(1→2)-arabinofuranosidic linkages in a ratio of 3:2, produced 42.5% inhibition of the precipitin reaction between antiserum and the whole purified polysaccharides. This is strong evidence that carbohydrate antigenic determinants capable of reacting with specific antibody but apparently incapable of inducing delayed hypersensitivity skin reactions were present on

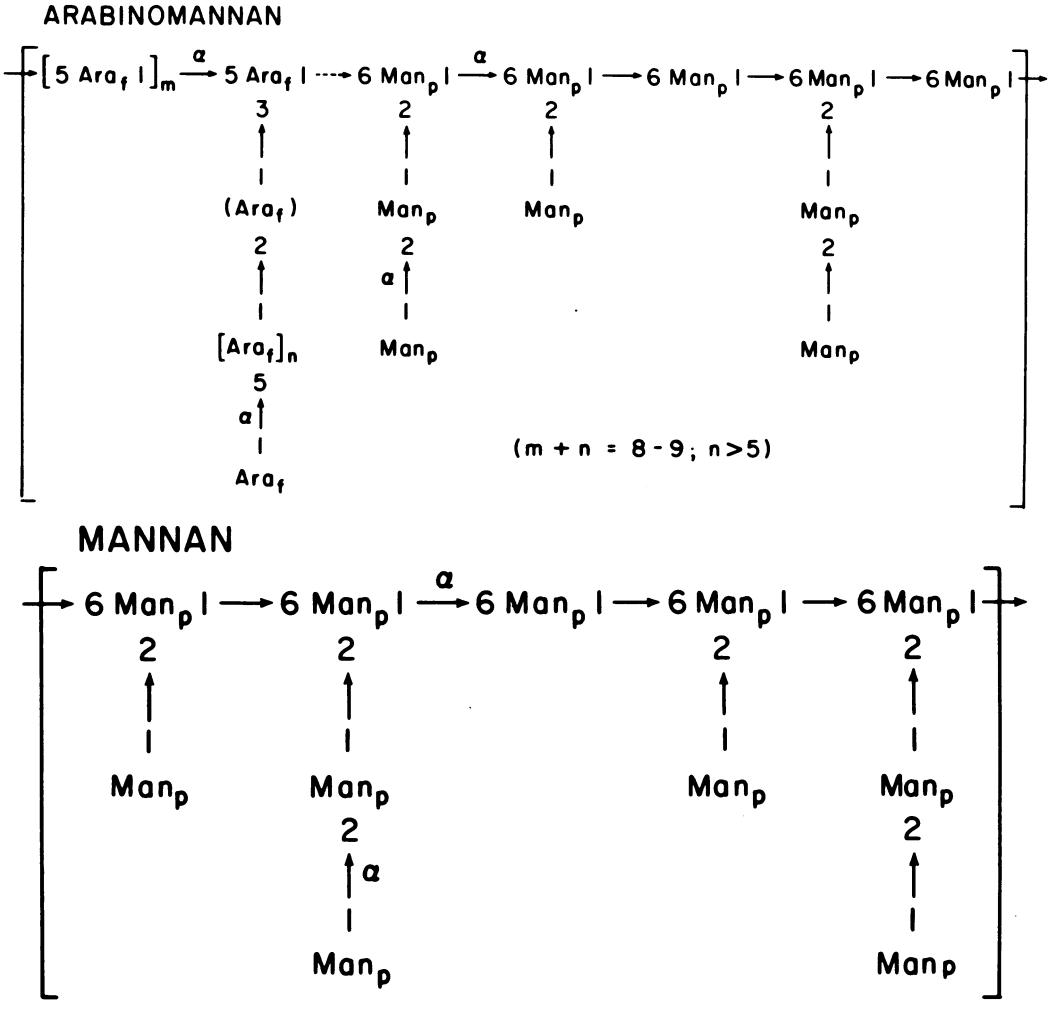

FIG. 6. *Proposed structure of M. tuberculosis arabinomannan (upper) and mannan (lower). Mannose (Man) and arabinose (Ara) residues are indicated as in their furanose (f) or pyrannose (p) forms, and the linkages are shown. The similarity of the major arabinose side chain of arabinomannan and of the side chain arabinogalactan (Fig. 5) should be noted. The similarity of the mannose side chains of arabinomannan and mannan is also noteworthy. These side chains probably vary among different mycobacterial species. Illustration kindly provided by Akira Misaki, Osaka, Japan.*

the arabinose-containing side chain of the original arabinogalactan molecule. Kotani and his co-workers reported similar observations with arabinose-rich polysaccharides and polysaccharide-glycopeptide complexes obtained from *M. tuberculosis* cell walls by enzymatic digestion (116).

The arabinogalactans and arabinomannans studied by Azuma et al. (11-15, 17, 205-207) were also capable of eliciting immediate skin reactions, serological reactions, and passive cutaneous anaphylaxis, but they were incapable of eliciting delayed skin test reactions. Polysaccharide antigens isolated by Wayne from *M. kanasasii*, *M. gastri*, and *M. marinum* by extraction with phenol, methanol, and acetone were found to react in immunodiffusion systems with antisera, and antigens were shared among the three species (199). Tsumita et al. demonstrated that the ability of antigenic polysaccharides to sensitize erythrocytes for passive hemagglutination reactions was dependent upon the presence of a lipid moiety within the sensitizing antigen preparation (193).

With the possible exception of concanavalin A-purified arabinomannan (49), a protein-free mycobacterial polysaccharide preparation has not been demonstrated to elicit delayed skin test

reactions to this date. However, the possible role of polysaccharide antigens in such reactions should not yet be discounted, especially in species other than man. Campbell showed that a purified polysaccharide derived from *Ascaris lumbricoides* would elicit delayed skin test reactions in rabbits (35). Similarly Gerety et al. have shown that pneumococcal polysaccharide may induce and elicit delayed skin test reactions in guinea pigs (81). However, Janicki and Aron (99) were unsuccessful in attempts to generate a humoral response in guinea pigs to electrophoretically isolated mycobacterial polysaccharides. Grappel used a synthetic polymannan completely devoid of nitrogen and emulsified with complete Freund adjuvant (containing heat-killed mycobacterial cells) to sensitize guinea pigs (90). Typical delayed skin reactions could be elicited in these animals with the synthetic polymannan.

Affinity Chromatography

Affinity chromatography is a purification method based on highly specific, dissociable interactions between two macromolecules. It is among the most versatile and powerful purification methods available in the field of immunochemistry, but substances are isolated on the basis of molecular parameters very different from those used in standard physicochemical procedures. That is, molecules are recovered together that have a given biological activity in common, even though they may have very different physical properties. Thus, when the isolation is made from lysed or physically disrupted cell walls, it is reasonable to expect great physical heterogeneity. In general, affinity chromatography methods can be expected to produce little or no denaturation or loss of antigenicity in contrast to harsh chemical extraction procedures. Molecular subunits or peptide or carbohydrate moieties, for example, can be expected to remain attached to their parent molecules. These subunits may be completely extraneous to the antigenicity of the product, or they may carry significant antigenic determinants.

Following the observations by Daniel and Wisnieski (63) and by Goldstein and Misaki (87) that mycobacterial polysaccharides react reversibly with concanavalin A, Daniel used absorbents of concanavalin A-agarose to purify one concanavalin A-nonreactive and two concanavalin A-reactive polysaccharides from *M. tuberculosis* culture filtrates (48). These polysaccharides were antigenic with respect to their ability to react with goat and guinea pig antisera in immunodiffusion and hemagglutination systems and with respect to their ability to elicit immediate and delayed skin test hypersensitivity reactions in guinea pigs and delayed reactions in a tuberculous monkey (49). When purified by concanavalin A chromatography, they contained variable amounts of protein, but their antigenicity did not correlate well with the protein content and was not abolished by treatment with proteolytic enzymes. Weak reactivity was observed in unsensitized, control guinea pigs (but not control monkeys), and it was thought that this reactivity was due to low levels of hypersensitivity resulting from environmental contact with other mycobacteria.

In subsequent studies, Daniel and Todd demonstrated that these three polysaccharides were present in filtrates from 12 different species of mycobacteria (62). Animals heterologously sensitized with one of three species of mycobacteria other than *M. tuberculosis* gave equal immediate and delayed skin test reactions (T. M. Daniel, R. C. Good, and S. D. Chaparas, unpublished data), providing an in vivo demonstration that these polysaccharides were widely distributed among mycobacteria.

Daniel and Misaki subjected these polysaccharides to carbohydrate analysis by gas and gas-liquid chromatography (61; A. Misaki and T. M. Daniel, unpublished data). They demonstrated that the concanavalin A-nonreactive polysaccharide was arabinogalactan with molar sugar ratios identical to those reported by Misaki and co-workers (142) for arabinogalactan prepared by alkaline extraction of mycobacterial cell walls. This material was known to identify with antigen 2 of the reference nomenclature. The concanavalin A-reactive polysaccharide of highest affinity was demonstrated to be arabinomannan of composition identical to that prepared by chemical extraction (139, 140, 154). This material was known to be antigen 1. The concanavalin A-reactive polysaccharide of low affinity was found to be a mixture of several polysaccharides, probably including one of much higher arabinose content than any previously described. These findings provide definite identification of immunoelectrophoretically recognized antigens 1 and 2 (104) with well-characterized, chemically purified polysaccharides. Contaminating nonantigenic glucan was found to be present in each of the concanavalin A-purified preparations.

In addition to concanavalin A, specific antibody can be used to prepare absorbents for affinity chromatography. Turcotte demonstrated that tuberculin-active material could be recovered by dissociating antigen-antibody precipitates formed from bacillary cell extract and rabbit antisera (194). Subsequently, Daniel used immunoabsorbents prepared with specific antisera for the purification of mycobacterial anti-

gens (52–54). Using this technique, Daniel and Anderson were able to recover highly purified antigen 5 (53, 54). Antigen 5 was found to be a protein with minimal or no sugar content and an amino acid composition consistent with a cytoplasmic origin. It had a sedimentation constant of 2.0S and a molecular weight of 28,500 to 35,000, and it was stable to 2-mercaptoethanol. When tested in sensitized guinea pigs, it was found to be equipotent with PPD in eliciting delayed skin test reactions and more species specific than PPD. In subsequent studies these same investigators (T. M. Daniel and P. A. Anderson, unpublished data) were able to purify antigen 6 by the use of an immunoabsorbent. Thus purified, it had physical properties similar to those found with chromatographically isolated material (58) and a greater skin test potency. Depending on the availability of monospecific antisera for use in preparing immunoabsorbents, immunoabsorbent affinity chromatography may offer a significant new dimension to mycobacterial antigen preparation.

Ribosomal Antigens

Baker et al. have studied ribosomes isolated from *M. smegmatis* and *M. bovis* protoplasmic extracts and found them capable of eliciting immediate and delayed skin test reactions and inhibiting in vitro macrophage migration in sensitized guinea pigs (21, 22, 126). Greatest skin test activity and specificity was found in the 30S subunit pool. These preparations contained 37% protein, and immunoelectrophoretic analysis suggested that five antigens of the reference culture filtrate (104) were present in a crude ribosomal preparation from *M. bovis*. Removal of ribosomal protein increased the potency and specificity of the ribosomes. The eluted protein also elicited delayed skin test reactions. Ortiz-Ortiz et al. also found ribosomal protein to be a potent delayed skin test antigen (157). Although they convey immunity against infection, ribosomes themselves do not serve as immunogens for the induction of delayed hypersensitivity (215).

Antigenic Peptides

In general, complete antigenicity is found only in molecules of fairly large molecular size. Antigenic determinants, however, may exist on smaller molecules. Thus haptens are simple substances and act as single antigenic determinants that can be demonstrated by appropriate inhibition techniques; they also can elicit skin test reactions but cannot serve as complete antigens. As has been noted above, certain oligosaccharides derived from cell wall polysaccharides have been shown to contain mycobacterial antigenic determinants (142). The relationship of molecular size to antigenicity of tuberculin components has been studied by Chaparas and his colleagues (41, 45). In general, smaller constituents were found to be less antigenic than larger ones in vivo and in vitro.

Yamamura and his many co-workers have studied a product of mycobacterial cell or cell wall extraction that they have termed tuberculin-active peptide (16, 145, 155, 182–184, 207–209). It has a molecular weight of 5,000 to 10,000. Even when given with an adequate adjuvant containing mycobacterial lipid, it did not induce either delayed or immediate hypersensitivity (209). However, as a purified material it was capable of eliciting delayed skin test reactions (183, 184, 207–209). It did not react in most immediate hypersensitivity assay systems, but could be coupled to erythrocytes for use in passive hemagglutination assays (182). From these observations, it can be concluded that this peptide contained at least one antigenic site. Moreover, this work clearly demonstrated that delayed skin tests can be evoked with mycobacterial products which, like haptens, are inadequate to serve as immunogens or complete antigens in other systems.

Stottmeier et al. prepared protoplasmic peptides from several species of mycobacteria by disruption of cells in a cell press, ultracentrifugation, and gel filtration (24, 189, 190). Their product had a molecular weight of 4,000 to 5,000. It was felt to be a glycoprotein with a peptide to carbohydrate ratio of 9:1. It probably contained 3 to 4 residues of glucose, 12 residues each of aspartic and glutamic acid, and smaller amounts of six other amino acids (189). It proved to be active in the eliciting of delayed skin test reactions in sensitized animals and to have greater specificity in this regard than the corresponding PPD preparation from the same species of mycobacteria (190).

In an early study, Misaki and his colleagues (144) studied mucopeptides isolated from mycobacterial cell walls. They also found glutamic acid, alanine, and diaminopimelic acid to be the major amino acids present. On the basis of enzymatic degradation studies of their product, they concluded that the antigenic reactivity present in their product was probably due to contaminating protein rather than a property of the peptide itself. A number of other investigators (25, 121, 141, 201) have also subjected cell walls to amino acid analysis and found α,ϵ-diaminopimelic acid (DAP), alanine, and glutamic acid to predominate; however, the antigenic reactivity of these materials was not examined. Peptides that were derived from cell wall peptidoglycan differed strikingly from protoplasmic

peptides and proteins containing large amounts of aspartic acid in addition to alanine, glutamic acid, and smaller amounts of many other amino acids (117, 189).

Kotani and co-workers degraded cell walls of *M. tuberculosis* enzymatically and purified the products of digestion by gel filtration and ion-exchange chromatography (116). In this fashion they isolated a tetrapeptide identified as L-Ala-D-Glu-DAP-D-Ala and a tripeptide identified as L-Ala-D-Glu-DAP. Polymeric forms of these peptides were also recovered. A family of peptidoglycans consisting of these peptides complexed to polysaccharides of arabinose, galactose, and mannose was found to be reactive with rabbit antisera. Their studies did not establish whether the antigenicity of these peptidoglycans rested with the polysaccharide or peptide portions of the macromolecules.

IMMUNOCHEMISTRY AND IMMUNOBIOLOGY

Molecular Heterogeneity

Before considering further the immunology of mycobacterial antigens, it is important to take cognizance of the molecular heterogeneity undoubtedly present in these antigens. All of these materials are derived from physically disrupted or lysed cells, and there is no a priori reason to expect that their release is a uniform process. Many antigens are probably derived from cell walls where repeating units abound, and individual antigenic determinant sites may be expected to be present on various-sized fragments in varying combinations and numbers. Thus, physicochemical homogeneity and immunological purity may be vastly different parameters in these materials. The observations of Kotani and associates (116) are consistent with this view.

The attenuated arcs seen on immunoelectrophoresis of mycobacterial extracts, as shown in Fig. 1, are strong evidence for molecular heterogeneity with variably charged molecules sharing the same antigenic determinant. Janicki and his co-workers (102) demonstrated this directly, showing that re-electrophoresis of a single fraction of an electrophoretically purified antigen resulted in constant and reproducible migration of each portion of the component. This conclusion can be inferred from the DEAE-cellulose chromatography studies of Kniker (113, 114), which demonstrated the extension of individual antigens through several eluted peaks. It is reasonable to assume that a similar heterogeneity exists with respect to molecular size.

With respect to mycobacterial polysaccharides, molecular heterogeneity is certainly great. The variability of molar ratios of sugars identified in various polysaccharides attests to this (12, 207). Clearly, the side chains of arabinomannan vary among species and perhaps strains of mycobacteria (139). The demonstration by Daniel that two polysaccharides of different concanavalin A affinities possess the same antigenic determinant (48) indicates that these molecules contain some portions that are identical and contain antigenic sites and other portions that are dissimilar and contain concanavalin A-reactive sites. Recent studies (Daniel, unpublished data) of a culture filtrate of *M. szulgai* suggested that antigens 1 and 2, usually readily separable, were present on a single polysaccharide molecule.

Thus, it may be unreasonable to expect real success in antigen purification using physicochemical techniques. Very-high-resolution techniques such as acrylamide gel electrophoresis may present particular problems because they may resolve antigenically homogeneous materials into many components differing slightly in the physical structure of nonantigenic moieties. If the desired goal is antigenic purity and homogeneity, then techniques such as affinity chromatography may offer more rational approaches to mycobacterial antigen purification.

Antigenic Specificity

It is possible to make a number of broad inferences concerning the specificity of mycobacterial antigens. It is quite clear that all unpurified mycobacterial extracts contain multiple antigens. Polysaccharide antigens, consisting principally of arabinogalactan and arabinomannan, are present in most of these, including OT and PPD, and are difficult to remove by physicochemical means. Chemically similar polysaccharides have been demonstrated to be present in many species of mycobacteria and in nocardia and corynebacteria, and the antigenic determinant of arabinogalactan (antigen 2) has been found to be shared among these various organisms (10, 13, 28–30, 62, 73, 74, 142, 163). It seems likely that the arabinose side chain of arabinomannan is responsible for this shared antigenicity. There is also long-standing indirect evidence based upon hemagglutination techniques that mycobacterial polysaccharides are antigenically cross-reactive with staphylococcal polysaccharides as well (131).

Minden and his co-workers, using radioiodinated antigen-binding techniques, have been able to demonstrate cross-reactivity between antigens of *M. bovis* BCG and antigens of many apparently unrelated bacteria (134, 135). Since the antigen used in these studies was iodinated, it can be inferred that the nonspecificity observed was related to protein or peptide components. Among the most striking observations

made on the cross-reactivity of mycobacterial antigens is the demonstration that line 10 guinea pig hepatocarcinoma (31, 34, 136) and human malignant melanoma (137) cells share antigenicity with *M. bovis* BCG.

The immunobiological significance of mycobacterial antigen nonspecificity certainly deserves further study. With respect to mycobacterial polysaccharides themselves, which are most likely the principal nonspecific antigens, an antigenic role with respect to antibody-mediated immunological phenomena seems well established. As noted elsewhere, the role of these polysaccharides in cellular responses is less clear. Especially when complexed to peptides, as is undoubtedly the case in nature, they probably deserve consideration as potential sources of nonspecificity in delayed hypersensitivity reactions.

In contrast, it is likely that some—although most certainly not all—mycobacterial proteins are species-specific antigens. The ion-exchange chromatographic studies of Kniker and LaBorde (114) and of Diena and co-workers (68) support this hypothesis. Similar conclusions can be drawn from studies using polyacrylamide gel electrophoresis, especially that of Minden and Farr (133). Turcotte absorbed mycobacterial protoplasmic extracts with antisera to heterologous mycobacterial species and removed most of the nonspecific reactivity (195). The absorbed material contained multiple components demonstrable by immunoelectrophoresis and acrylamide gel electrophoresis. The recent studies of Janicki, Chaparas, and several collaborators (unpublished data) have demonstrated skin test specificity in homologously and heterologously sensitized guinea pigs and found this specificity to be associated with acrylamide gel electrophoresis fractions containing principally antigens 5 and 6.

Recent immunodiffusion studies (57; T. M. Daniel and L. S. Todd, unpublished data) have shown that antigen 6 contains at least two antigenic determinants, one of which appears to be specific for *M. tuberculosis* and the other of which is present in several other mycobacteria. It is tempting to relate this observation to that of Yoneda and Fukui, who reported similar findings for the antigen they designated as alpha (78). Moreover, when the physical properties and isolation procedures described by Yoneda and Fukui (79, 210–212) are compared with those of Daniel and Ferguson (58), whose a_2 protein is known to be antigen 6 (60), it seems likely that the alpha antigen is in fact antigen 6. Norlin et al. made similar observations with an antigen designated gamma in *M. gastri*, and they stated that this antigen identified immunologically with the alpha antigen of Yoneda and co-workers (153). Ridell and Norlin demonstrated similar reactions of partial identity between several species of nocardia and mycobacteria for an antigen they designated alpha but which is clearly distinct from antigen 6 (166); it is reasonable to suspect that this antigen is cell wall arabinogalactan. It must be recognized that the demonstration of both specific and nonspecific antigenic determinants on the same molecule of mycobacterial protein poses additional challenges to those scientists interested in mycobacterial antigen purification.

Antigen 5 has been demonstrated to be limited to *M. tuberculosis* and *M. bovis* among 14 species of mycobacteria (Daniel and Todd, unpublished data). Its mobility when electrophoresed in acrylamide gel is not similar to that of the specific antigen described by Minden and Farr (133). The beta protein antigen of Yoneda et al. (213) was also found to be highly specific, but it is not possible to relate this antigen to other described proteins. The L-asparaginase purified by Hornez and co-workers (95) from *M. phlei* was found to have immunological identity with L-asparaginase isolated from *Escherichia coli*. In contrast, mycobacterial beta-lactamase (109) and catalase (67, 188, 200) are at least partially species specific.

There is considerable reason to suspect that mycobacterial constituents of relatively low molecular weight and rapid electrophoretic mobility may have a high degree of species specificity. As discussed previously, the glycoprotein of Minden and Farr (133) isolated by gradient acrylamide gel electrophoresis is such a substance. Roszman and co-workers (172) also noted species specificity in a mycobacterial component moving rapidly in acrylamide gel, but they did not isolate sufficient material to allow its characterization. It is likely that only relatively small proteins survive heat denaturation and are present in PPD, and they are probably responsible for specificity present in this product. The PPD fractionation studies of Moulton and his collaborators (147) and of Nagai and his colleagues (148) demonstrated the ability to elicit delayed skin tests to be associated with rapidly moving components. In the latter studies (148), a molecular weight of approximately 10,000 was estimated for the isolated tuberculin-active material.

The observation by Stottmeier et al. that the 4,000- to 5,000-molecular-weight peptide that they isolated possessed significant species specificity (190) is noteworthy because the amino acid composition, although not the sequence, of this peptide was determined. It seems likely that the first identification of the structure of a my-

cobacterial antigenic determinant site will come from studies of small-molecular-weight peptides containing tuberculin activity.

Immunological Responses to Mycobacterial Antigens

Clearly, many constituents of mycobacteria are antigenic. Indeed, since under natural circumstances these materials are always introduced into a host in the presence of mycobacterial cells with adjuvant properties, a high degree of responsiveness to mycobacterial antigens might be anticipated. There is evidence that, as in leprosy, the immunological response in patients with tuberculosis may pursue one of two pathways. In most patients, delayed cellular hypersensitivity predominates. In a small number of patients with disseminated disease, humoral responses predominate and there is a corresponding failure of cellular responses (27).

Naturally occurring mycobacterial infections of both man and lower animals are associated with readily demonstrable circulating antibodies to mycobacterial antigens (27, 55, 56, 76, 133, 159). The extensive literature on this subject will not be included in this review. It should be noted that antibodies to polysaccharide antigens are most readily demonstrated by agar gel precipitation techniques in patient sera (105), but more sensitive assays have readily demonstrated antibodies to both protein and polysaccharide antigens (55, 56). Serodiagnostic approaches to tuberculosis and other mycobacterial diseases generally have been of little use because of false positive reactions obtained in noninfected individuals (55, 56, 76). These results probably are due to circulating antibodies against nonspecific mycobacterial antigens resulting from host environmental contact with nonpathogenic organisms. The availability of species specific antigens for use in these assays might offer a significant new impetus to the use of serodiagnostic techniques in mycobacterial diseases.

There is little question that experimental animals develop circulating antibodies to mycobacterial proteins and polysaccharides under conditions of hyperimmunization, and scores of investigators have prepared reagent antisera in animals for use with mycobacterial antigens. Similarly, circulating antibodies have been demonstrated to various mycobacterial antigens, both protein and polysaccharide, under conditions of experimental immunization or infection. It is beyond the scope of this review to consider this large subject, except to point out that the nature of the antibody response observed has been found to be related more to the intensity of stimulation than the specific nature of the antigen used (47). A notable exception is the finding of Janicki and Aron (98) that serological reactivity in young guinea pigs to mycobacterial antigens, predominantly polysaccharides, decreased as the immunizing dose of heat-killed tubercle bacilli was increased.

With respect to cell-mediated immunological responses, there is general agreement that mycobacterial proteins induce hypersensitivity under natural and appropriate experimental conditions. As noted, purified mycobacterial proteins evoke delayed skin test reactions in sensitized hosts and are reactive in a variety of in vitro assays of cellular hypersensitivity. The reactivity of mycobacterial fractions in various assays of cell-mediated immunological responses is presented in Table 3. When PPD is used as the test antigen in cell culture studies, the results may be somewhat clouded by the observation that PPD in large doses may serve as a nonspecific mitogen for human (152) and animal (191, 214) B lymphocytes, an observation probably not due to contaminating lipopolysaccharide (191, 192). In mice, unheated *M. tuberculosis* culture filtrate may be similarly nonspecifically mitogenic (192). Similarly, in nonsensitized guinea pigs PPD may stimulate the production of migration inhibition factor (214).

The antigenic reactivity of mycobacterial polysaccharides in cell-mediated immunological responses deserves special consideration (Table 3). It is clear that chemically extracted and highly purified polysaccharides cannot elicit delayed skin test responses, even when used in large amounts. In contrast, less rigorously treated polysaccharides, which may still contain labile nitrogenous antigenic determinants, do elicit delayed skin test reactions and induce inhibition of macrophage migration (18–20, 40, 49, 86). This antigenic reactivity was not reduced by treatment with proteolytic enzymes (19, 20, 49, 86) and did not correlate quantitatively with the amount of protein present (49). The methods used for purification may have major influence on polysaccharide antigenicity. Additionally, the work of Baer and his co-workers (19, 170) suggests that guinea pigs may react to mycobacterial polysaccharide antigens that do not elicit delayed hypersensitivity responses in man. Finally, there is the interesting dichotomy that Chaparas and co-workers observed between cultured lymphocyte mitogenesis and production of migration inhibition factor (44, 45). Mycobacterial polysaccharides may have very distinctive immunobiological properties, and they deserve further study.

CONCLUSIONS AND FUTURE PROSPECTS

All species of mycobacteria contain multiple

TABLE 3. *Comparison of reactivity of mycobacterial antigens in cell-mediated hypersensitivity responses*

Test system	Seibert fraction (176)[a]				
	Polysaccharide I	Polysaccharide II	Protein A	Protein B	Protein C
Immunoelectrophoresis (reference antigen content) (104)	1, 2 (51)	3 (51)	1, 2, 4, 5, 6 (51)	1, 2, 5, 6, 7 (51)	2, 6, 7 (51)
Reactivity in vivo					
Skin test					
Man	Negative (127, 176)	Negative (176, 180)	Positive (197)	Positive (197)	Weakly positive (176, 197)
Guinea pig	Negative (15, 142, 176, 207) Positive (19, 42, 45, 49, 60)	Negative (176, 180)	Positive (18, 20, 40, 42, 45, 60, 147, 176)		
Tuberculin shock					
Guinea pig	Positive (101)			Negative (101)	
Reactivity in vitro					
Lymphocyte mitogenesis					
Man	Negative (60, 93, 103)	Negative (93, 103)	Positive (60, 103)	Positive (103)	Positive (103)
Guinea pig	Negative (44)			Positive (44, 45)	
Macrophage migration inhibition					
Guinea pig	Positive (42, 44, 45, 86, 92)	Negative (42, 44, 45, 92)		Positive (42, 45)	

[a] In preparing this table, the authors have interpreted the preparations of various investigators as containing one or more of the principal components of Seibert's fractions on the basis of their described properties.

antigens. Cell wall (but perhaps not cytoplasmic) polysaccharides, proteins, and peptides all have been shown to be antigenic in some circumstances. With respect to polysaccharides, the ability to elicit delayed hypersensitivity reactions remains in doubt and may be limited only to guinea pigs. Arabinogalactan and arabinomannan are excellent antigens in serological systems, and the antigenic determinant of the former has been identified with a major arabinose side chain. This determinant is probably shared by all species of mycobacteria and by nocardia and cornynebacteria as well. Although providing a useful approach to the study of mycobacterial cell walls, immunological studies of arabinogalactan are unlikely to advance either the taxonomy of mycobacteria or diagnostic techniques used for mycobacterial diseases. Arabinomannan, however, deserves further study, for the side chains of this polysaccharide probably vary among mycobacterial species. It seems reasonable to hypothesize that this polysaccharide might be responsible for the surface antigen specificity which permits specific seroagglutination of many mycobacterial strains.

Probably all cytoplasmic mycobacterial proteins have antigenic potential, and under most conditions of host infection or immunization, immunological responses probably develop to many of them. Some, but not all, of these proteins are species specific. Many peptides, both of cytoplasmic and cell wall peptidoglycan origin, also carry antigenic determinants. Greater research effort has been expended on the study of protein than polysaccharide or peptide antigens, and much of this work has focused on attempts to isolate and purify them. At the present time this research has not yet reached the goal of producing specific and readily standardizable antigens, nor are most of the physicochemical techniques that have been used likely to do so. Yet the work which has been done by many investigators gives strong evidence that such antigens are within reach. Imaginative research should be ready to move forward to this goal in the immediate future.

Standard physicochemical techniques are based upon the physical properties of molecules and may have limitations because of the molecular heterogeneity inherent in available mycobacterial products. In this context it is probably important to distinguish clearly between purity as judged by molecular physical homogeneity and antigenic purity as represented by the presence of a single, specific antigenic determinant site. Newer techniques such as affinity chroma-

tography may provide a feasible approach to achieve antigenic purity. Species specific antigens have been recognized in mycobacteria. One realistic goal would be to define the chemical nature of specific antigenic determinants and seek to obtain specific antigens whose antigenic purity could be measured chemically against this knowledge and whose physical status could be separately assessed by appropriate physical techniques. Perhaps, as a more distant goal, synthetic antigens with known mycobacterial specificity could be prepared.

The need for purified and well-standardized antigens is great. Not only are they necessary for accurate clinical diagnosis of tuberculous infection, but also mycobacterial antigens serve as standards in the assessment of cell-mediated (T lymphocyte) immunological functions for a variety of clinical and fundamental biological situations. Truly purified and standardized highly specific mycobacterial antigens should contribute broadly to our knowledge of major biomedical problems.

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